EMBRYONIC STEM CELLS ALTER CARDIOMYOCYTE ELECTROPHYSIOLOGICAL PROPERTIES

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Priyanka Karan

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EMBRYONIC STEM CELLS ALTER CARDIOMYOCYTE

ELECTROPHYSIOLOGICAL PROPERTIES

Approved by:

Dr. Samuel C. Dudley Jr, Advisor
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Robert Butera
School of Electrical Engineering
Georgia Institute of Technology

Dr. Micheal E. Davis
School of Biomedical Engineering
Georgia Institute of Technology

Date Approved: [Date Approved by Committee]

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LIST OF ABBREVIATIONS

AP	Action Potential
APD	Action Potential Duration
CM	Conditioned Media
CV	Conduction Velocity
Cx43	Connexin43
ESC	Embryonic Stem Cell
FGF	Fibroblast Growth Factor
FPD	Field Potential Duration
g_j	Gap Junction Conductance
HF	Heart Failure
G_{Na}	Maximum Conductance of the Na^+ Current
hESCs	Human Embryonic Stem Cells
HSCs	Hematopoietic stem cells
IM	Incubated Media
ISI	Interspike Interval
LR	Luo-Rudy
LVAD	Left Ventricular Assist Device
MSCs	Mesenchymal Stem Cells
MEA	Microelectrode Array
Na^+	Sodium
NRVM	Neonatal rat ventricular myocytes
RT-PCR	Real Time Polymerase Chain Reaction
SCN5A	Sodium channel, Voltage-gated, Type V, Alpha subunit
VEGF	Vascular Endothelial Growth Factor

SUMMARY

Heart failure (HF) is a disease affecting millions of people worldwide. Currently with treatment options that do not address the underlying loss of myocardial cells, doctors and researchers have been relentlessly trying to find alternative treatments. With the advancement in stem cell biology, one of the emerging therapies for CHF is cellular cardiomyoplasty. The usual response to cardiac injury is replacement of the dead tissue with fibrotic scar. Cellular cardiomyoplasty theorizes that introducing stem cells to the injured region leads to cardiac regeneration and improvement in function. Embryonic stem cells (ESCs) are one source of progenitor cells for cellular cardiomyoplasty because of their potency and availability. Nevertheless, the consequences of ESC application are unknown, and previous studies have suggested that other progenitor cells may be arrhythmogenic. Therefore, we studied the electrophysiological implications of ESC-mediated therapy by evaluating the effects ESCs have on neonatal rat ventricular myocytes (NRVMs).

To mimic expected in vivo engraftment rates, 5% mouse ESCs were co-cultured with NRVMs. Field potential duration (FPD), interspike interval (ISI) and conduction velocity (CV) were measured using a multi-electrode array system (MEA). Comparing cultures without and with 5% ESCs at 4 days, the mean bipolar FPD of NRVMs increased from 26.3 ± 2.2 ms (n=10) to 44.3 ± 6.2 ms (n=9; $p < 0.05$), the ISI increased from 358.3 ± 62.8 ms (n=10) to 947.8 ± 214.6 ms (n=7; $p < 0.01$), and CV decreased from 14.2 ± 1.3 cm/s (n=8) to 4.6 ± 1.2 cm/s (n=5; $p < 0.01$). These initial findings led to further evaluation of whether ESCs were having direct or paracrine effects on NRVMs.

Media conditioned by $3x10^6$ ESCs for 24 h was diluted 1:1 with fresh media (ESC CM) and then introduced to NRVM cultures on the day of plating. CM was changed daily and altered mean FPD, ISI, and CV to 46.1 ± 7.8 ms, ISI to 682.0 ± 128.5 ms, and 4.2 ± 0.4 cm/s (n=8; $p < 0.01$ for each measure), respectively at 4 days as compared to media treated similarly but with exposure to ESCs.

Cardiac sodium (Na^+) channel and connexin43 (Cx43) expression were studied as possible explanations for the observed decrease in CV in the presence of ESCs. Western blots revealed an increase in phosphorylated Cx43/unphosphorylated Cx43 ratio by 96% in the 5% mouse ESCs and 53% ESC CM as compared to controls (n=8; $p < 0.01$ for each). Also, cardiac Na^+ channel expression was significantly decreased to 64.9 ± 6.0% (n=8) and 73.8 ± 13.8 % (n=7) of the control for ESCs and ESC CM respectively. RT-PCR revealed a similar reduction in cardiac Na^+ channels. Using single cell patch clamping techniques, a 38% reduction in cardiac Na^+ current was measured. Also, there was a significant decrease in peak Na^+ current in ESC CM-treated cardiomyocytes. In addition cardiac simulations were conducted based on experimental Na^+ current and Cx43 changes. These simulations revealed a similar decrease in CV.

In summary, the presence of ESC altered the electrical properties of native heart tissue, prolonging CV and APD. These effects were mediated by soluble factors secreted by ESCs and resulted, in part, from reduced Na^+ channel and Cx43 levels. These effects may need to be considered when designing cardiac cell replacement strategies.

CHAPTER 1

INTRODUCTION

1.1 The Epidemic of Heart Disease

Today, even with the advent of modern therapies, heart disease still remains a leading cause of death worldwide. According to the Center for Disease Control and Prevention, almost 23.5 million Americans suffer from heart disease in some form, leading to over 700,000 deaths a year and making it the number one killer of both men and women in America.[2] As heart disease patients, many Americans suffer from a heart attack each year and often develop a condition called heart failure (HF).

Ironically, while modern therapies have prolonged the life of cardiac patients, these therapies have also led to more cases of end stage HF.[3] Each year approximately a quarter million people die from HF.[4] The disease is responsible for over 11 million doctors' visits and 3.5 million hospitalizations yearly.[5] Unfortunately, in the past 25 years, this is the only category of heart disease that has increased in terms of prevalence, hospitalization rate, and cost.[6] In fact, the number of deaths from heart failure has increased 6-fold in the past four decades and is the leading cause of hospitalization in seniors over 65 years old.[7] Annually, it is estimated that $25-40 billion are spent for the care of heart failure patients.[5] This makes the cost of hospitalization for heart failure twice as much as that for all forms of cancer combined.[5] All these statistics make it clear that HF as a disease is widespread.

HF occurs most commonly when the heart's ability to pump blood through the body is limited. A failing heart's inability to pump decreases blood flow out of the heart, which causes venous return to back up and manifest as tissue congestion.[8] HF may have deleterious effects on the renal, respiratory and hepatic organ systems. Patients suffering from HF are at a risk for heart rhythm disturbances (arrhythmias), and approximately 50% of all deaths related to HF result from arrhythmias.[9]

1.2 Current Treatments and Limitations

While lifestyle changes are used for primary or secondary prophylaxis, most patients with HF are dependent on using medications. Useful medications include diuretics, inotropes, vasodilators, and beta blockers. Despite treatments with these drugs, almost all HF patients will experience at least one acute episode that requires hospitalized treatment. Studies have shown that twenty percent of patients hospitalized will experience repeat episodes and that fifty percent of those patients end up visiting the hospital again in only six months.[10]

One of the major limitations in management of HF is that therapies do not address the underlying cause of myocardial dysfunction. For example, the inability of the heart to pump blood efficiently often results in reduced kidney function and increased vascular tone. Drugs like diuretics affect the kidney, while vasodilators affect arterial tone. These drugs improve the effects of heart failure rather than the actual condition of the heart. These drugs also have adverse effects such as electrolyte depletion and hypotension in the case of diuretics[7] and dizziness in the case of vasodilators.[11]

Studies have shown that the organ systems mentioned above are linked through a network of neurohormones. These hormones, like epinephrine, stimulate the heart to beat faster and stronger in order to compensate for the inefficiency of the heart caused by HF. Doctors generally prescribe angiotensin converting enzyme inhibitors (ACE-I) and beta blockers to reduce the deleterious effects of increased neurohormones. Nevertheless, ACE-I drugs have drawbacks: they cannot completely prevent the body from manufacturing angiotensin[12] and they can lead to hypotension.[7]

Other treatment options like biventricular pacemakers or left ventricular assist devices (LVADs) are available to treat HF.[13] LVADs can augment or replace myocardial function but are generally not considered a permanent solution because of the limitations of power supply, increased risk of infection, and increased risk of thrombosis. In addition, implantation is expensive and complicated. Cardiac transplant is possible but is limited by the number of available organs and a 20% 1-year mortality rate.[14]

Because of these shortcomings, new methods of treatment are needed. Alternative drug therapies, permanent artificial hearts, and xenotransplantation are currently being researched. Another promising therapy is cellular cardiomyoplasty.

1.3 Cellular Cardiomyoplasty- A Promising Treatment Option

An alternative approach to mechanically assisting or transplanting a damaged, dysfunctional heart is to generate ‘‘new’’ functional myocardium. These strategies include induction of transdifferentiated adult cells to replace dead myocardium, attraction of endogenous progenitor cells, and addition of exogenous progenitor cells. These approaches have been collectively termed cellular cardiomyoplasty. Often these techniques are assisted by addition of factors designed to encourage cardiac cell growth and differentiation.

The effects of various growth factors on cardiomyocyte proliferation have been studied extensively. Factors such as basic fibroblast growth factor (bFGF),[15] insulin-like growth factor (IGF-I, II),[17] transforming growth factor beta-1 (TGFβ-1)[16] and platelet-derived growth factor (PDGF) [17] have been associated with cardiomyocyte proliferation. Cardiomyocyte mitogenic factors have also been under investigation with the hope of converting post-mitotic stage cardiomyocytes into proliferating cells.[20, 21] While research in mitogenic factors is promising, it still has not lead to the conversion of terminally differentiated, post-mitotic, myocardial cells into proliferating, regenerative cells.

A possible alternative solution would be converting non-contractile tissue into contractile myocardium through a process known as transdifferentiation. Murry et al.[22] were able to inject a virus carrying the myoD gene into rat myocardial scar tissue and demonstrated the subsequent production of contractile proteins. Nevertheless, there is no evidence that these transfected cells can form contracting myocytes, and the transfection rate was reported to be as low as 14%.[22] Another overriding risk is the tumor potential of these vectors which may limit their clinical application.

Potentially salvaging the remaining peri-infarct zone is another area of intense research interest. The non-contractile scar tissue is usually surrounded by an area of hibernating myocardium and being able to restore blood flow to this region may contribute to helping systolic function. Angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and bFGF, have been shown to induce the development of collaterals and increase capillary density in ischemic canine myocardium.[23] An alternative approach to the use of angiogenic factors in restoring regional blood flow is to use transmyocardial revascularization, which uses laser- or needle-created channels to induce an inflammatory response that leads to the subsequent increase in regional blood flow, probably by stimulating angiogenesis. Both of these approaches are aimed at salvaging any viable, functional myocardium. It is still not known if these approaches produce a clinically significant increase in myocardial function.

Another area that is being researched is the application of cells that might assist the myocardium in generating a contractile force. In vitro animal studies have demonstrated that transplanted cardiomyocytes within a normal or infarcted myocardium remain viable for months and have the ability to proliferate and differentiate in situ.[18] Other studies have shown the presence of intercalated disks and connexin 43 (Cx43), a gap junction maker required for cell-to-cell electrical coupling, within grafted tissue.[25, 26] The graft has shown spontaneous contraction with fetal cells or after electrical stimulation when implanted with myoblasts.[19] These results are promising, but an ideal cell source for the job has not yet been established.

1.4 Possible Sources for Cell Therapy

Several distinct cell types have been used in experimental and pilot trials. The question of which cells are best suited for cardiac regeneration and vascularization has not been answered yet, however. Researchers are looking at neonatal cardiac myocytes, skeletal myoblasts, mesenchymal stem cells (MSCs), and embryonic stem cells (ESCs), among others.

Cardiomyocyte transplantation is a promising approach for treating HF. After transplantation of embryonic rat ventricular myocytes, studies have reported a trend for increased vascularization in an infarcted model with survival of transplanted cells lasting up to 53 days.[28] Transplanting ventricular myocytes into dilated cardiomyopathic hamsters improved ex-vivo contractile performance one month after transplantation.[29] An investigation by Reinecke et al. revealed that neonatal cells expressed the cadherens junction proteins N-cadherin and Cx43, indicating potential integration into host tissue.[25] Most long term follow-up investigations in infarct models reported that integration of engrafted cells appeared to be hindered by scar tissue, thus separating the transplant from the host.[20] In addition, the availability of such a cell source would be limited, if available at all, when used in a clinical application and an appropriate immunosuppressive regime would have to be designed.[20]

Skeletal myoblasts were investigated because, unlike fetal cells, their use does not involve issues like ethics, availability, and immunogenicity. Also, they are resistant to ischemia, the cause of most myocardial injury. Research on rabbit myocardium demonstrated that transplanted autologous skeletal myoblasts improved myocardial performance after 6 weeks.[21] Data from pressure-volume loops indicated that engrafted myoblasts improved global systolic function.[22] N-cadherin and Cx43, which were expressed in myoblast cultures, were down-

regulated after implantation, however.[33] This study suggests that problems could arise during the integration of the graft with the surrounding host tissue.

Increasing evidence suggests that progenitor cells of the adult organisms have a capacity to give rise to several lineages and may be suitable for regenerative medicine.[23] These adult cells are present in most organs and act as a reserve for replenishing cells that die. Depending on their tendency to adhere to tissue culture plates and their surface markers, adult progenitor cells from the bone marrow can be categorized into hematopoietic stem cells (HSCs), endothelial progenitor cells, MSCs, or side-population cells.[24] For example, multipotent adult progenitor cells have been isolated from bone marrow and can differentiate into visceral mesoderm, neuroectoderm, and endoderm in vitro.[36]

While research is being conducted using all these cell types, currently the most promising results come from MSCs. An innovative study published in Nature showed the potential of adult bone marrow cells to improve injured myocardium.[37] Cells obtained from transgenic male mice were injected into the healthy myocardium adjacent to the infarct site. In only 9 days, a band of myocardium was observed in the treated mice as compared to the control mice.[37] In contrast, other studies have shown no evidence of MSC-derived cells transdifferentiating into cardiomyocytes after transplantation.[38]

ESCs are the most primitive cells and have the ability to develop into all of the unique cell types that make up the human body.[25] Derived from the inner mass of the blastocyst, these cells may be harder to isolate compared to their adult counterparts but are potentially more beneficial because of their totipotency and ability to proliferate without differentiating. The later property facilitates cellular expansion and addresses the need for large cell numbers required in

therapeutic uses. ESCs are currently the only cell source that can provide ex-vivo an unlimited number of cardiac cells for transplantation. In theory, they can be extensively characterized and genetically engineered to promote desirable characteristics such as resistance to ischemia or improved contractile function to cater to specific applications.[26] Researchers are looking at ESC derived cells as gene therapy vehicles aimed at modifying the local myocardial environment by secreting paracrine factors.[26]

1.5 Potential of ESCs as a Cell Source for Cardiac Regeneration

Research has established that murine ESC (mESCs) can give rise to cardiomyocytes both in vitro and in vivo.[27,28] Klug et al.[27] transplanted mESCs-derived cardiomyocytes into the left ventricular wall of mice and found that after transplantation, the donor cells displayed cardiomyocyte phenotype. These implanted cells formed stable intracardiac grafts and displayed gap junctions. The selected cardiomyocyte cultures were more than 99% pure and highly differentiated. The genetically selected cardiomyocytes formed stable grafts in adult mouse hearts for 7 weeks after implantation. This study demonstrated the potential of grafts from ESC-derived cardiomyocytes to survive in normal tissue with adequate vascularizaton.[27]

In one study, green fluorescent protein (GFP) expressing, cultured ESCs were injected into an infarcted rat heart. Within six weeks, there was significant improvement in cardiac function as compared to the controls.[29] Immunostaining against GFP, cardiac sarcomeric alpha-actin, alpha-myosin heavy chain, or troponin I, experiments confirmed the survival and differentiation of engrafted cells. The shape and size of the transplanted GFP-positive cells were similar to the host cardiomyocytes. These studies indicate that transplanted ESCs survived and differentiated in injured myocardium and improved cardiac function. Similar differentiation patterns have also been seen in the tissue culture studies using human ESCs (hESCs).[30]

Other studies have looked at the longer-term benefits of ESC transplantation on cardiac function.[45] GFP expressing mESCs were implanted into the border region of the infarct area. After 32 weeks, researchers noticed increased survival in the MI rats with ESC transplantation. Hemodynamic and echocardiographic data showed significant improvement of cardiac function. GFP-positive tissue was indentified in the infarcted region and showed the presence of several

cardiac proteins. These data suggest that ESC grafts exhibit long-term survival capabilities and may contribute to the long-term improvements in heart function.

The percentage of ESCs that differentiate into cardiac myocytes is low (typically only 1% of a differentiating culture).[31] Due to the large number of ESCs needed for clinical applications, it is important to develop a streamlined process where a large number of cells can be differentiated quickly in vitro, especially in terms of developing pure cultures of myocytes for cardiac therapies. Studies are being done to investigate ways to increase the number of cardiac progenitor cells from ESCs using approaches like retinoic acid, inhibition of bone morphogenic protein (BMP) signaling, and modulating Wnt signaling.[32, 33]

Some research groups have looked into implanting undifferentiated ESCs into the myocardium. While studies have shown undifferentiated ESCs have signaling mechanisms that cause cardiomyocyte differentiation and lead to improved cardiac function,[34] these cells also have a high risk for developing into teratomas.[35] The tendency to form teratomas is a major limitation for the use of ESCs for human cardiac repair. Another limitation is immune rejection. Possible options to overcome this problem include stem cell banks to reduce donor-recipient differences, immunosuppressive therapy, and creation of "universal" donor cells by genetic manipulation.[36]

1.6 Cell Replacement Therapies and Arrhythmic Risk

The previous sections discussed the variety of cell sources that are currently being investigated as replacement sources. Unfortunately, no consensus has been reached on what is the optimal cell source. This is partially because of the possibility of arrhythmic risk demonstrated by experimental and clinical trials. Arrhythmias may offset some of the benefits associated with cell replacement. Therefore, many studies are looking at potential risk associated with cell therapy options.

Over the past 2 years, a number of early-phase clinical studies have started addressing the question of safety in cardiac cell transplantation. These studies have predominantly used skeletal myoblasts and bone-marrow-derived stem cells. In 2001, autologous skeletal myoblasts were expanded ex-vivo and injected into the post infarcted scar. After 5 months, there was improved contraction in the native myocardium.[37] These results suggested that the improved function of the scarred area was probably related to the engrafted myoblasts. While these results seemed promising at the time, a follow up study in 2003 found an occurrence of arrhythmias in many patients in this clinical trial.[38] The arrhythmias could have been due to differences in the expression channels of skeletal myoblasts and cardiomyocytes. Other potential causes include an inflammatory response to the exogenously applied cells.[39] An alternative theory is that reentry pathways could be created by cell-independent, injection-related disorganization in the border area of the infarcted region.[53]

In 2005, Abraham et al.[55] published results that offered new information regarding arrhythmic risk associated with skeletal myoblast transplantation. Using an optical mapping system, the group studied the electrophysiological effects of skeletal myoblasts on a 2-

dimensional (2D) cardiomyocyte culture. They demonstrated that skeletal myoblasts did not electrically couple well with the neighboring cardiomyocytes. The skeletal myoblasts increased the occurrence of reentrant circuits in these 2D cultures. In their studies, they also demonstrated that only by induced expression of Cx43 they were able to reduce the arrhythmic risk.[55]

These results suggest the need for further investigation into the electrical effects of cell replacement therapies. The Abraham et al.[55] study suggests that skeletal myoblasts are unable to fully integrate into the surrounding myocyte culture and develop contractile function. The alteration in the action potentials in skeletal myoblast/cardiomyocyte co-cultures may be related to myoblast secreted paracrine factors. More research into the effects of the Cx43 expressing skeletal myoblasts needs to be conducted. Given the reduced electrical coupling of skeletal myoblasts, embryonic stem cell derived cardiomyocytes may be the most likely cell source for cardiac regeneration.[45]

1.7 Mechanisms of Arrhythmias

Ventricular arrhythmias are commonly seen in people suffering from HF. Three fundamental mechanisms exist for arrhythmias: triggered activity, automaticity, and reentry. Triggered activity occurs when an action potential (AP) incites a subsequent premature beat (Figure 1-1).[40] Automaticity refers to the ability of cardiac cells to spontaneously and repetitively depolarize in the absence of external stimulation (Figure 1-2).[41] Reentry is continuous electrical activity facilitated by slow conduction (Figure 1-3).[40] These mechanisms underlie sudden deaths resulting from heart failure.

Afterdepolarizations and Triggered Activity

Triggered activity arising from either early afterdepolarizations (EADs) or delayed afterdepolarizations (DADs) is one of the mechanisms that initiates arrhythmia. Triggered activity is caused by oscillations in the membrane potential that follow the upstroke of an AP. EADs take place during repolarization of the membrane potential, while DADs occur after completion of repolarization (Figure 1-1). When these oscillations are large enough to hit threshold, they invoke a series of premature APs.[42] DADs are facilitated by tachycardia, whereas EADs occur when the AP is prolonged, as occurs in bradycardia.

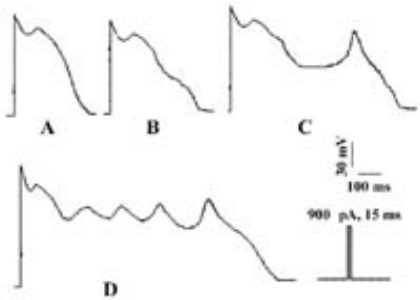

Figure 1-1 – Triggered Activity. In panels A-D, show voltage oscillations known as early afterdepolarizations (EADs) as the AP prolongs. The EADs can eventually reach threshold and lead to triggered spontaneous APs. (Picture: Li Y et al.[1])

Automaticity

Another mechanism of initiation of ventricular arrhythmias is automaticity. Automaticity occurs from a gradually decreasing membrane potential, which creates an AP upon reaching threshold (Figure 1-2). When automaticity arises from cells with normal negative diastolic potential, it is called "normal automaticity," whereas "abnormal automaticity" arises from partially depolarized cells.

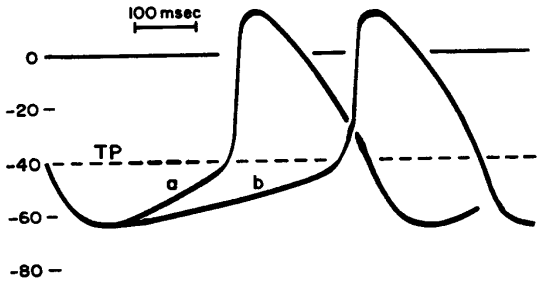

Figure 1-2 – Automaticity. In abnormal automaticity, the rate depolarization may be increased or the threshold potential (TP) reduced, that is, the level that triggers the rapid phase 0 depolarization is more negative. This would result in a tachycardia. (Picture: www.lhsc.on.ca/uwodoc/pages/arrmech.htm)

The electrophysiologic changes that favor automaticity are a decrease in inward rectifying current I_k, an increase in the pacemaker current, or a combination of both. Studies found that the pacemaker current, I_f, is activated at less negative potentials in failing human ventricular cells.[43]

Reentry

Reentry is a mechanism of maintenance for ventricular arrhythmias.[42] Reentry takes place when an impulse returns to excite previously activated myocardial cells that have already been repolarized and are in phase 4, the resting potential state.[60] Heart tissue that has been depolarized for long periods provides the basis for the development of reentry. Conditions favoring reentrance are slow conduction, short refractory periods, large dispersion of refractory periods and the presence of a unidirectional block.[42]

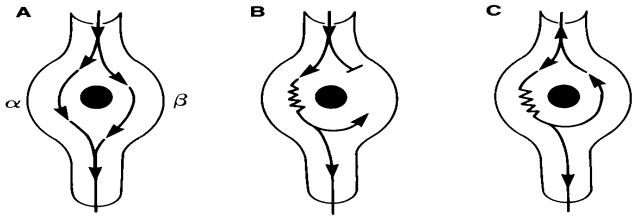

Figure 1-3 – Reentry. A classic form of reentry known as "circus movement reentry" (A) A particular piece of tissue has two potentially conducting pathways (α & β). During sinus rhythm, the depolarization wave front passes across the tissue, depolarizing both pathways. (B) An event, usually an extrasystole, exposes the presence of the two pathways since they may have slightly different conduction velocities and recovery rates. The wave front blocks in β but conducts slowly down the α pathway, in this case, because the β pathway was refractory. (C) By the time the impulse reaches the distal end of the α pathway, the β pathway has recovered sufficiently such that the impulse can conduct retrogradely up the β pathway and back down the α pathway. A reentrant tachycardia is established. This mechanism is dependent on slow conduction being present. (Picture: www.lhsc.on.ca/uwodoc/pages/arrmech.htm)

As previously mentioned, slowed conduction is the primary cause for reentry circuits. Gap junctions play a pivotal role in the CV and signal propagation of cardiac tissue.[44] Gap junctions are clusters of closely packed channels, which allow for direct connection of cytoplasmic compartments of neighboring cells. They allow the passage of ions and small molecules.[45] To form a gap junction, each of the two cardiomyocytes contributes a connexin hemichannel. Connexin hemichannel are made by oligomerization of six connexins, which are transmembrane protein molecules. Connexin 43(Cx43) is the most abundant connexin in the heart and has been found in most parts of the heart except in cells of the sinoatrial and atrioventricular nodes.[46] Studies have suggested that Cx43 may be the most important determinant of myocardial conduction in ventricular cells like NRVMs.[47] Research has also shown that Cx43 phosphorylation plays an important functional role in gap junction activity and in the membrane assembly.[48]

Connexins are not the only proteins responsible for CV. In the heart, voltage-gated sodium (Na^+) channels determine the amplitude and slope of the AP, which is especially important when it comes to controlling the impulse CV and in maintaining excitation waves.[49] Dysfunctional Na^+ channels have been shown to be responsible for several inherited cardiac electrical disorders, such as Long QT and Brugada syndromes, potentially leading to fatal arrhythmic events. A recent study, discussed how minor genetic alterations in Na^+ channels that lead to a loss of current are enough to cause arrhythmic disorders.[67] For example, isolated cardiac conduction disorder is characterized by slowed intramyocardial conduction, and results in a loss of Na^+ current. This can cause individuals to experience bradycardia and syncope resulting from delays in ventricular excitation. [50]

1.8 The Arrhythmogenic Potential of ESCs

As scientists are looking at the benefits of cellular transplantation, there is also an increased concern for possible downside risks such as the potential arrhythmic risk. Arrhythmic risk is known to be inversely proportional to cardiac function. Since cell therapy results in improved function, one important question is whether cellular cardiomyoplasty will suppress an arrhythmic tendency by improving cardiac function or whether will it further add to the electrical heterogeneity, thereby enhancing any arrhythmic predisposition.

Stem cell-derived cardiomyocytes have displayed arrhythmic characteristics in vitro.[51] CMs derived in vitro showed AP heterogeneity, maintained automaticity for long periods and had prolonged AP durations. The prolonged AP durations showed easily inducible triggered electrical activity. These findings have important clinical implications, as automaticity and triggered activity are known fundamental mechanisms of arrhythmias. hESC-derived cardiomyocytes also show similar behavior.[52]

1.9 Specific Aims

Cellular cardiomyoplasty is being considered as a possible therapy for cardiac regeneration. While numerous sources exist, ESCs are one source of progenitor cells for cellular cardiomyoplasty because of their potency and availability. Although potent, the risk factors associated with ESC application are unknown. Previous studies have shown the arrhythmogenic potential of in-vitro ESC-derived cardiomyocytes, but studies have not looked at the electrophysiological changes in native cardiomyocytes caused by the introduction of ESCs, however.

There are three main specific aims for the project:

1. *Do ESCs cause alterations in cardiomyocyte electrophysiology?*
2. *What is the underlying ionic basis of any observed changes?*
3. *How do ESCs affect these changes in cardiomyocytes?*

CHAPTER 2

TOOLS AND METHODS

Hodgkin and Huxley were the first to study the electrical activity of a squid axon by voltage-clamping and obtained the first intracellular recording of an AP.[53] The development of the patch clamp, which allows one to study single ion channel changes, provided a better understanding of the molecular mechanism of cardiac AP generation.[54] Both of these techniques were limited because they did not allow assessment of CV and lacked the ability to conduct long-term measurements, which are essential for studying the efficacy of drug treatments, cell-based therapies, and other treatment options.

2.1 MEA - a Tool for Electrophysiology Measurements

The multi-electrode array (MEA) system overcomes these limitations by recording simultaneous extracellular potentials from a monolayer of cells deposited on top of its detection surface. The MEA is a glass dish with 60 titanium nitride coated gold electrodes arranged in a two-dimensional (8 X 8) configuration on the floor of the dish (Figure 2-1). The relatively small dimensions of the electrodes and precise positioning capabilities support high spatial resolution recordings and can be helpful in the analysis of the activity of complex, multi-cellular systems. The minimal invasiveness of the MEAs also allows for long term cell recordings.[73] Each electrode records extracellular electrical signals of aggregated cardiomyocytes called field potentials (FP).[55]

Although the FP components are not identical to the intracellular AP, they can provide useful information about the effects of various electrophysiological changes on the excitation generation and propagation in multi-cellular preparations of cardiomyocytes.

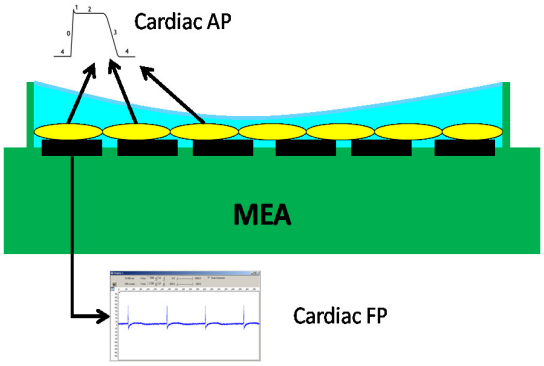

Figure 2-1- Schematic of the MEA culture dish. Cells are cultured in a glass MEA dish lined with 60 electrodes (black) spaced at 200 µm in an (8x8) arrangement. Cardiomyocytes (yellow) attach to the bottom of the dish, where they generate individual cardiac action potentials (AP). Each electrode records the cardiac field potential, which is the combination of the signals generated by each cell.

MEA Measurements and Their Significance

Three fundamental mechanisms exist for arrhythmias: automaticity, reentry and triggered activity. MEA measurements record information about FPD, ISI, and CV. These measurements can be useful in assessing arrhythmic risk because they can provide information regarding the underlying mechanisms.

The FP measured by an MEA can be analyzed in terms of the minimum, maximum, and duration (Figure 2-2). FP duration is the time between the initiation of depolarization and the end of repolarization. Halbach et al. demonstrated that FPs recorded from multi-cellular preparations of myocytes contain information about APD and individual transmembrane currents (Figure 2-3).[1] Thus, characterization of the FP allows researchers to estimate AP changes and the contribution of individual current components to the AP non-invasively.[1]

The ability to estimate AP is important in cardiac research because increases in AP duration can lead to triggered activity. Since MEAs can provide duration information, one can compare FP durations of various cultures. Given that a relationship between AP to FP exists, it is possible to study the effects of a cardiomyocyte/ESC co-culture condition on FP duration and consequently AP duration with an MEA system.

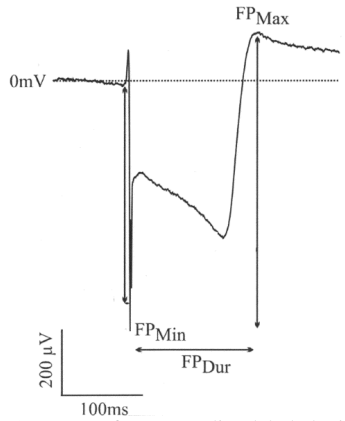

Figure 2-2 – A representative FP. A FP waveform recording labeled with FP minimum (FP_{Min}), FP maximum (FP_{Max}), and FP duration (FP_{Dur}). (Picture: Halbach et al.[1])

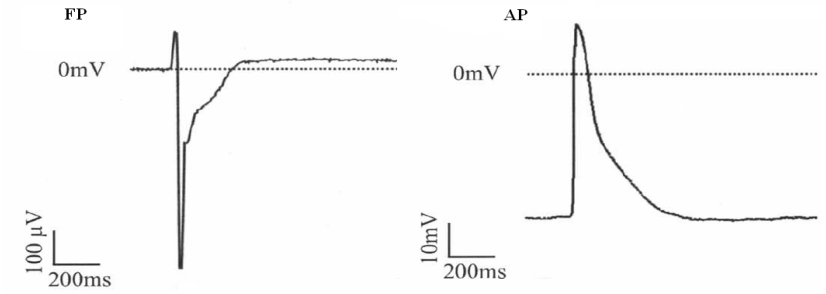

Figure 2-3 – FP to AP correlation. Simultaneous measurements of FPs and APs recorded from multi-cellular preparations of spontaneously active isolated embryonic mouse cardiomyocytes. Linear relationships between the AP rise time and duration and the FP rise time and duration exist. (Picture: Halbach et al.[1])

Interspike interval (ISI), the inverse of the spontaneous beating rate of the in vitro cardiomyocyte culture, can also be obtained from MEA recordings. These measurements are significant because ISI is a measure of automaticity (Figure 2-4).

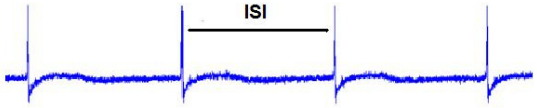

Figure 2-4- ISI Measurement. FP recording taken over a one second time interval from a single electrode. The ISI is labeled.

While the MEA cultures are too small to assess reentrant arrhythmias directly, they can be used to determine the CV, a strong determinant of this type of arrhythmia. Since MEAs can measure signals from multiple electrodes simultaneously, it is possible to measure the CV as an excitation wave moves through the culture. CV is the speed at which the electrical signal travels through cell culture (Figure 2-5). CV provides information about cell-to-cell communication, which is reflected by depolarization and repolarization characteristics of the AP. Information about gap junction remodeling can also be obtained from the CV. Structural changes of gap junctions can predispose the myocardium to arrhythmias, and studies have shown that as CV decreases, the Cx43 density in cultures also decreases.[75] In general, a decreased CV correlates with an increased possibility of reentry.

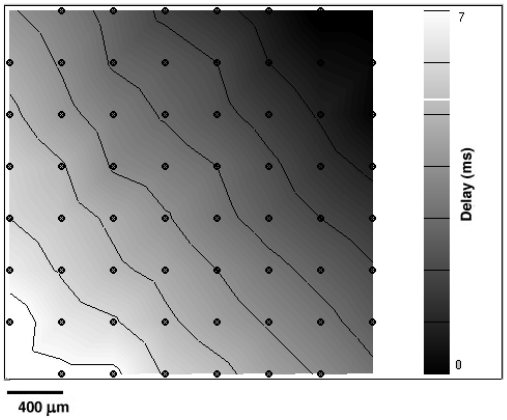

Figure 2-5 – Representative activation map for a culture. The site of earliest activation is initiated in the upper right hand corner and spread through the culture. The grayscale gradient depicts isochrones of activation. Similarly in the human heart, APs are generated in a single area (sinoatrial node) and spread through the atria by cell-to-cell contact. CV can be calculated using activation times and isochrones for direction.

2.2 Methods and Techniques

Embryonic Stem Cell Culture

For all the experiments conducted, the ESCs were isolated from the mouse ESC R1 cell line (ATCC, Manassas, VA). The R1 ESCs were seeded onto feeder layers grown in T-25 flasks. The ESC media consisted of high-glucose Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA) supplemented with 15% FBS, 200 mmol/L L-glutamine, 5 X 10^{-5} mol/L β-mercaptoethanol, 10 mmol/L non-essential amino acids, 5000 U/mL penicillin/streptomycin, 50 mg/mL Leukemia Inhibitory Factor. ESCs were expanded for 48h and then split to prevent differentiation.

When the cells were not used in experiments, they were centrifuged, put in dimethyl sulfoxide freezing media (Sigma-Aldrich, St. Louis, MO) and stored for the first 24 h at -70°. After 24h, they were transferred into liquid nitrogen. When needed, these cells were thawed and re-suspended in ESC media and grown as normal. Cell numbers and viability were measured using a hemacytometer and trypan blue exclusion test. In the trypan blue exclusion test, a cell suspension is mixed with a dye and then visually examined to determine whether cells take up or exclude the dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

Neonatal Rat Cardiomyocyte Isolation and Culture

Ventricular cardiomyocytes were isolated from 2 to 3 day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) using a isolation kit purchased from Worthington Biochemical Corporation (Lakewood, NJ). Isolations were preformed by the method described in Appendix A. Briefly, the beating hearts of anesthetized Sprague-Dawley rats were surgically removed and then immediately placed in a centrifuge tube containing 35 mL sterile calcium- and magnesium-free Hanks Balanced Salt Solution (pH 7.4). The suspension was incubated overnight at 4°C with trypsin (50µg/ml). On the following day, the tissue was treated with a trypsin inhibitor for 30 min, followed by collagenase for 45 min both at 37°C. The tissue was titrated and the supernatant was filtered through a cell strainer. Then, the cells were centrifuged at 1000 rpm for 3 min, and the cell pellet was re-suspended in cardiomyocyte media consisting of DMEM, 10% Fetal Bovine Serum (FBS), and 200µg/mL penicillin/streptomycin. After measuring cell yield and viability with the trypan blue exclusion test, cells were plated on tissue culture dishes for 1.5 h to allow the attachment of non-myocytes. The non-adherent cells were collected and recounted using a hemacytometer. Cells were plated onto an MEA dish, and the cultures were maintained at 37°C in a humidified incubator (95% air/5% CO_2). Unattached cells were removed by washing with Phosphate Buffered Saline (PBS) after 24 h. The medium was replaced daily, and all experiments were conducted within a week of cell isolation.

Neonatal Rat Cardiomyocyte Co-Culture with Embryonic Stem Cells

In the control group, consisting of neonatal cardiomyocytes only, cells were plated on an MEA at a seeding density of 2 X 10^6 per MEA. In the 5% ESC treatment group (5% ESC), 2 x

10^6 cardiomyocytes were co-cultured with 1 x 10^5 ESCs. All MEAs were pre-coated with 0.1% gelatin to allow for better adhesion.

Neonatal Rat Cardiomyocyte Co-Culture with ESCs Conditioned Media

To create ESC conditioned media (ESC CM), media bathing 2 x 10^6 ESCs for 24 h was removed and mixed with cardiomyocyte media 1:1 to avoid the cell apoptosis that is seen with undiluted CM. For the control and ESC CM groups, 2 x 10^6 cardiomyocytes were plated onto an MEA culture dish. The media was changed every 24 hours.

Western Blot Analysis

All cultures were washed in ice-cold phosphate-buffered solution at 96 h, harvested in a RIPA buffer consisting of 50 mmol/L Tris, 150 mmol/L NaCl, 1% Igepal CA-630 (NP-40) , 0.5% Na^+ deoxycholate, 0.1% SDS, 5 mmol/L EDTA, 1 mmol/L PMSF, 1 μg/mL phosphatase inhibitor (Sigma Chemicals) and 1 μg/mL protease inhibitor (Sigma Chemicals)**.** The cells were then scraped from the dish and the cells' membranes were disrupted by sonication for 2 min. Homogenate protein concentrations were determined by the Lowry protein assay (Bio-Rad, Hercules, CA). Protein (15 μg) was loaded in each well of a pre-casted 10% Tris-Glycine polyacrylamide gel (Cambrex Bio Science Rockland, Rockland, ME). The gel was run at 100 V for 1.5 h at room temperature and then blotted onto a nitrocellulose membrane for 1 h at 15 V. The membrane was blocked with 5% milk for 1 h and incubated with a rabbit polyclonal anti-Cx43 antibody 1:2000 dilution (Sigma Chemicals) in 5% BSA at room temperature for 1 h. The blot was further incubated with goat anti-rabbit HRP-conjugated secondary antibody (Sigma Chemicals) in 1:2000 dilution in 5% BSA for 1 h at room temperature and visualized using the ECL plus chemiluminescence kit (Amersham Biosciences, Piscatway, NJ). Tubulin or GAPDH

expression were used as loading controls and probed sequentially with rabbit monoclonal anti-Tubulin (Sigma Chemicals) or rabbit monoclonal anti-GAPDH 1:5000 (Sigma Chemicals) and goat anti-rabbit HRP-conjugated 1: 2000 secondary antibody (Santa Cruz Biochemicals, Santa Cruz, CA) in 5% BSA solutions. Cardiac Na^+ channel expression was targeted with rabbit monoclonal anti-cardiac Na^+ channel antibody (1:1000), which was generously provided by Dr. Peter Mohler from the University of Iowa. Tubulin was used as a loading control for the Na^+ channel protein studies. The intensity of the individual bands was quantified using ImageJ (NIH software, Bethesda, MD).

Reverse Transcription Polymerase Chain Reaction

The expression of the cardiac Na^+ channel protein scn5a was confirmed using quantitative real-time RT-PCR. Total RNA from untreated and treated cardiomyocytes was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) with the addition of RNase–free DNase I. Reverse transcription was carried out at 42°C for 30 min with iScript reverse transcriptase (Bio-Rad), 1 μg total RNA, and 4 μL of 5x iScript reaction mix following the manufacturer's instructions. The first strand cDNA was used as template for subsequent PCR reactions. Each PCR reaction contained 12.5 μL of IQ SYBR Green Supermix (Bio-Rad) and 2.5 μmol/L primer pairs in total 25 μL reaction volume. The forward primer rtPCRscn5aF (5' GAAGAAGCTGGGCTCCAAGA 3') recognized a sequence from exon 26. The reverse primer, rtPCRscn5aR (5' CATCGAAGGCCTGCTTGGTC 3'), was complementary to exon 27 of scn5a cDNA. The reactions gave rise to a 101 bp PCR product. All amplifications were performed in triplicate and consisted of 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C in a BioRad thermocycler iCycler. PCR products were analyzed by relative standard curve methods. β-Actin was used as a reference for making quantitative comparison.

Electrical Activity Recordings

Extracellular recordings from the cultured neonatal rat cardiomyocytes were performed using a MEA data acquisition system (Multi Channel System, Reutlingen, Germany). The MEA was connected to an amplifier system, which included a heat source. Simultaneous recordings of extracellular potential from all electrodes were performed at a sampling frequency of 10 kHz and a temperature of 37°C. Internal channel #15 was used as a reference electrode. Recordings were considered significant when at least 50% of the total electrodes on the MEA recorded depolarizations of -10 μV or greater. As described previously in Halbach et al., the data were analyzed off-line with a customized toolbox programmed for MATLAB (Mathworks, Natick, MA).[35] FP parameters were averaged for five predefined locations on the array, four in each quadrant and one in the center (Figure 2-6). CV was determined using the activation time and the distance between adjacent electrodes. The direction of signal propagation was taken to be normal to the isochrones. CV was calculated by taking the average velocity in three pre-specified directions of propagation. This was done to minimize the error resulting from any inhomogeneities in proprogation. The angle between the lines was set at 45°. CV is path independent in MEAs, and is based on the idea of uniform conduction, which may not be the case always.

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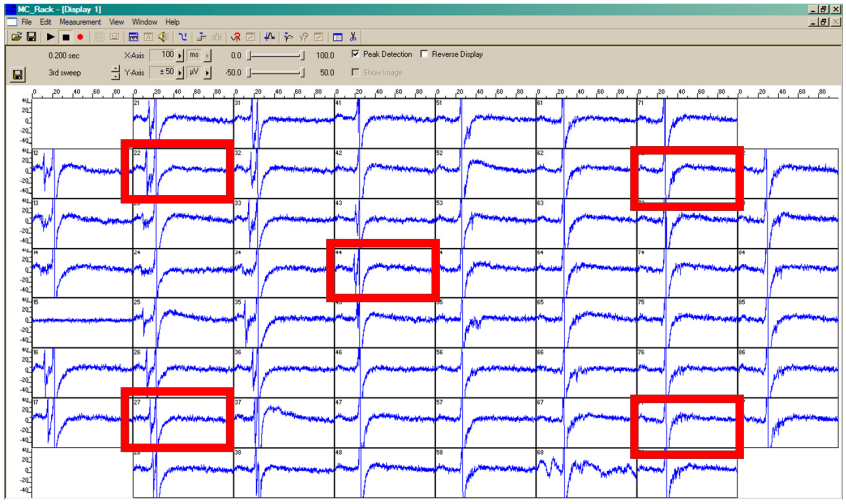

Figure 2-6 – Measurements of FP parameters. A screen shot of one second recordings of the FP from all electrodes with five representative electrodes highlighted in red. These five highlighted electrodes were the five most often used for determining the mean FP duration and ISI in a preparation. If sufficient signal was not available at these electrodes, new electrodes were chosen, which maintained the same geographic relationship.

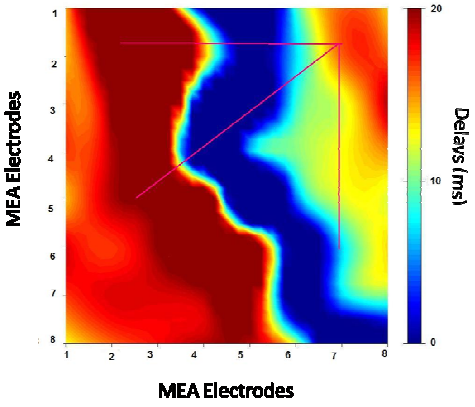

Figure 2-7 - CV determination in cultured NRVMs. The CV is calculated from the paths shown in pink. This map shows the time delays between pacemaker activation and activation at a given location on the MEA. Blue indicates the origin of the spread of excitation (the pacemaker site). The color gradient corresponds to time delays from pacemaker activation.

Whole-Cell Voltage-Clamp Electrophysiology

Whole cell patch clamping was used to determine the effects of changes in Na^+ channel protein expression on Na^+ current. Cardiomyocytes were plated onto plastic cover slips in 24-well plates on the day of isolation. There were two groups for these experiments: ESC CM and control. Glass pipettes were pulled on a Sutter Model P-97 horizontal puller to create a resistance of 1.5 to 2.5 MΩ. The glass pipettes were filled with a solution of (in mmol/L) CsCl 60, Cesium Aspartate 80, EGTA Na^+ 11, HEPES 10, Na_2ATP 5 and pH 7.2 with CsOH. The bath solution consisted of (in mmol/L) Na 30, N-methyl-D-glutamate chloride 100, CsCl 5, $CaCl_2$ 2, $MgCl_2$ 1.2, HEPES 10, Glucose 5 and pH 7.4 with HCl. Once a seal between the cell membrane and the pipette was established, a small amount of suction was applied to obtain whole cell current measurements. The holding potential of the cell was maintained at -100 mV. The voltage was stepped from -100 mV to +60 mV to test the presence of voltage-gated Na^+ channels. Voltage was increased at 10 mV intervals and currents were measured at each interval. In all recordings, 80% of the series resistance was compensated, yielding a maximum voltage error of ~1 mV. Data were sampled at 10 kHz and later filtered at 5 kHz for analysis. Currents were recorded and analyzed with an Axopatch 200B amplifier, Axon Digidata 1230A A/D converter and pClamp software (Molecular Devices Corporation, Sunnyvale, CA).

Calculations of the Decreases in CV

The simulations in this study were based on the Luo-Rudy (LR)[56,57] model, which is a theoretical model of the mammalian ventricular AP. In this model, the AP is constructed from the various ionic processes that were derived from experimental data obtained from studies performed on guinea pigs. The LR model was used to correlate in vitro changes in CV to changes in Na^+ and connexin concentration.

The LR model was used in two configurations: 1) a one-dimensional fiber consisting of 130 cells and 2) a 2D model consisting of 400 x 400 cells. In the first case, the theoretical fiber is composed of serially arranged ventricular cells. The LR model was used to compute the ionic currents and concentration changes, for each cell in the fiber. The details of the theoretical fiber are provided in an earlier publication.[58] Briefly, the temporal transmembrane current fluxes of the LR model are related to the spatial (axial) current flow by a finite difference approximation of the cable equation:[58]

$$\frac{V_{i-1}^{t} - 2V_{i}^{t} + V_{i+1}^{t}}{\frac{R_i \Delta x}{\pi a^2}} = [C_m \frac{V_i^{t+1} - V_i^{t}}{\Delta t} + I_{ion} + I_{stim}] 2\pi a \Delta x R_{CG}$$

Equation- 1

where I_{ion} represents the individual membrane ionic currents, I_s is the stimulus current density, a is the radius of the fiber, C_m is the membrane capacity, V^t_i is membrane potential at cell i and time t, Δx is the discretization element (in this case equal to the length of 1 cell), R_{CG} is the ratio between capacitive and geometrical areas, and R_i is the axial resistance per unit length. R_i is in turn composed of myoplasmic resistance (R_{myo}) and gap junction resistance (normal R_g = 1.5 ohm cm^2, which corresponds to a conductance of g_j = 2.5 microS). A g_j= 2.5 μS results in a CV of 56 cm/s, which is typical of propagation along the fibers. A g_j = 0.37 μS results in a CV of 14.3 cm/s (the CV value for the control group), which is typical of transverse propagation. The equation was solved for V^t_i using the Crank-Nicholson implicit method. Extracellular resistance and flux boundary conditions at the edges were neglected.

Calculations in the 2D model were made using the LR phase 1 model [56] instead of the LR phase 2 model for the sake of simplicity and computational efficiency.[57] A more detailed

description of the 2D model can be obtained in a previous publication.[59] Briefly, 2D tissue can be treated as a continuous system in which propagation can be modeled by a partial differential equation of the form:

$$\frac{\partial V}{\partial t} = -\frac{I_{ion}}{C_m} + D\left(\frac{\partial^2 V}{\partial x^2} + \frac{\partial^2 V}{\partial y^2}\right) \qquad \text{Equation- 2}$$

where V is the membrane voltage, D is the diffusion current coefficient and I_{ion} is the sum of ionic currents. Equation 2 was solved using a modified version of the Crank-Nicholson implicit method.

These simulations were performed by Dr. Prakash Viswanathan at the University of Pittsburgh. In these studies, the effect of Na^+ and connexin modulation on AP conduction was studied. An external stimulus was applied at specific start sites to initiate an AP (Figure 2-11). A stimulus was applied to cell 1 in the 1D model and to the middle of the culture at position (200,200) in the 2D model. CV was calculated by dividing the distance between the cells (40 x Δx) by the difference between the time that the upstroke velocity reaches a maximum at cell 80 and cell 40 in the fiber. A similar method was used in the 2D model for calculating CV. AP propagation was assumed to be uniform in all directions. The connexins were modeled as simple resistors between cells. The resistance of the connexins (R_g) leads to changes in the CV. The gap junction conductance (g_j) was chosen such that AP propagation was uniform in all directions. The initial values in the model were chosen to mimic a CV of 15.5 cm/sec, which is based on experimental control CV data. g_j was reduced by a fixed percentage to mimic the changes in connexin expression observed in experiments (Figure 3-8). Na^+ channel changes

were modeled by reducing the maximal conductance of the Na^+ current (G_{Na}) by fixed percentages (Figure3 -8).

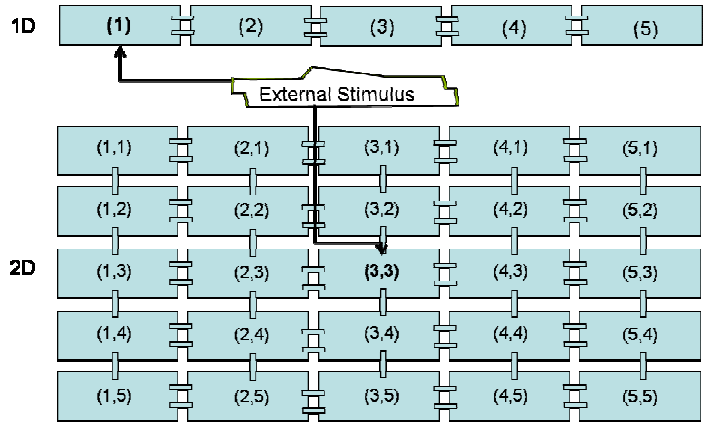

Figure 2-8 – A schematic of the cell grid used in the 1D and 2D simulations. An external stimulus was applied to cell 1 in 1D and cell (3,3) in 2D to initiate an AP.

Statistical Analysis

Patch-clamp data was obtained and analyzed using pClamp 8.0 (Axon Instruments, Union City, CA). (GraphPad Software, Inc., San Diego, CA). Differences in recordings were determined by analysis of variance. Differences between specific groups were seen using post-hoc testing. Data was presented as the mean ± SEM. A $p < 0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

Groups Studied:

Control – 2 X 10^6 Cardiomyocytes

5% ESC – 2 X 10^6 Cardiomyocytes and 1 X 10^5 mouse ESCs

ESC CM – 2 X 10^6 Cardiomyocytes with diluted 1:1 media from ESCs for 24 hours and cardiomyocyte media

IM –2 X 10^6 Cardiomyocytes with media kept overnight in an incubator

3.1 ESC Exposure Alters Cardiac Electrophysiology

Prolongation of FPD in Treatment Conditions

Cardiomyocytes that were exposed to ESCs for 96 h had a significantly longer FPD (44.0 ± 6.2 ms) as compared to the control (26.3± 2.2 ms, n=9) (Figure 3-1). In the ESC CM group, the FPDs increased (46.0± 7.8 ms, n= 8), which is significantly higher than the controls. The 5% ESC and ESC CM groups were not statistically different from each other. To ensure that the effects of the CM were not a result of overnight incubation, incubated media (IM) only experiments were conducted. The FPDs of IM were comparable to those of controls suggesting

that the results are due to the paracrine effect of ESCs.

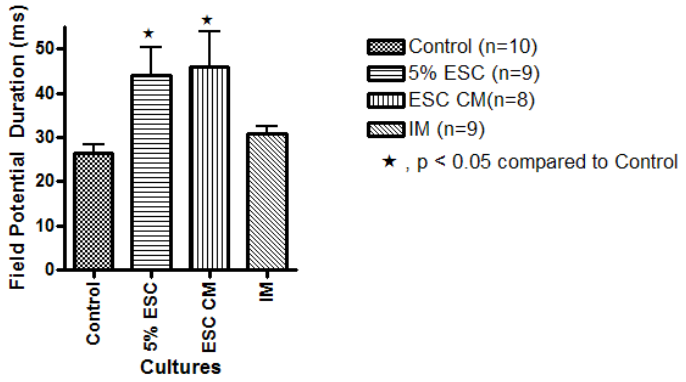

Figure 3-1- Mean FPD at 96h. Co-culture with ESCs prolongs cardiomyocyte FPD. The mean FPD ± SEM are shown for the control, 5% ESC, ESC CM, and IM groups at 96h. *, $p < 0.05$ as compared to control.

Prolongation of ISI in Treatment Conditions

ISI showed a 164% increase in the 5% ESC group as compared to the control (Figure 3-2). The ISI of the 5% ESC group was 947.8 ± 214.6 ms (n=7) and the ISI of the control group was 358.3 ± 62.9 ms. (n=10; $p<0.05$). The ESC CM group showed a similar trend with an ISI of 682.0 ± 128.5 ms (n=9). ISI in the IM group was not statistically different from the control.

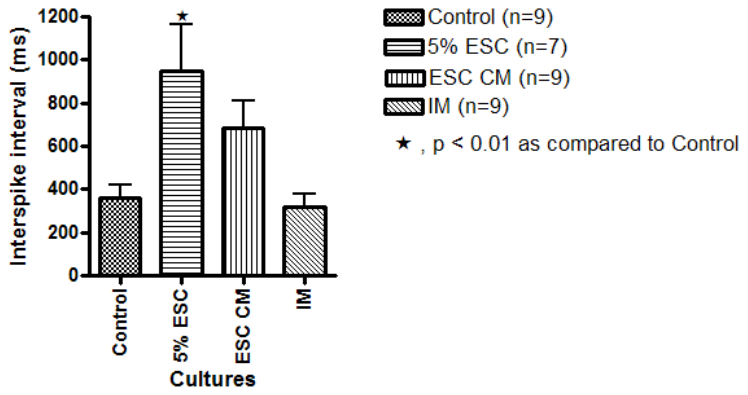

Figure 3-2- Mean ISI at 96h. Co-culture with ESCs prolongs cardiomyocyte ISI. The mean ISI ± SEM are shown for the control, 5% ESC, ESC CM and IM groups at 96h. *, $p < 0.05$ as compared to control.

Reduction in Conduction Velocity

ESC exposure resulted in a 62% reduction in CV as compared to controls (Figure 3-3). A similar reduction occurred upon exposure of cardiomyocytes to the CM. The CV for the control was 14.23 ± 1.27 cm/sec. The CV for the 5% ESC and ESC CM groups were 4.56 ± 1.29 cm/sec and 4.18 ± 0.40 cm/sec respectively. Both 5% ESC and ESC CM groups were statistically different from the control group. The IM group had values that were not significantly different from the control.

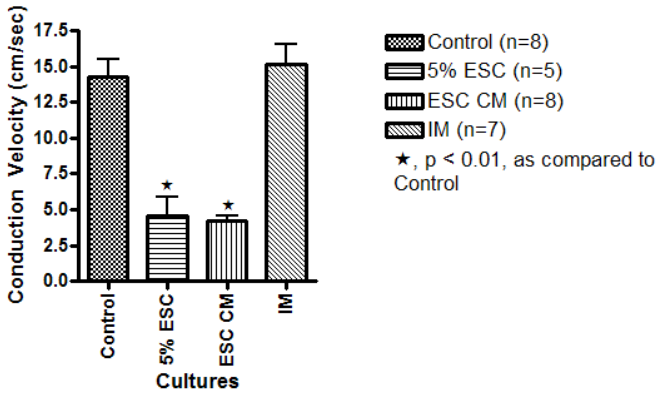

Figure 3-3- Mean CV at 96h. Co-culture with ESCs tends to prolong CV in cardiomyocyte co-cultures. The mean CV ± SEM are shown for the control, 5% ESC, ESC CM and IM groups at each time point. *, $p < 0.01$ as compared to control cultures.

3.2 ESC-Mediated Reductions in Na^+ and Cx43 Channels May Explain the Changes in Cardiomyocyte Behavior

ESC Co-Culture and Conditioned Media Conditions Reveal a Decrease in Connexin43 Expression

No differences in total Cx43 expression were observed between treatments and controls (Figure 3-4 A). A distinct difference in phosphorylation of Cx43 was seen in the treatments as compared to the controls (Figure 3-4 B). Western blots suggest that changes in the phosphorylation state of Cx43 upon ESC exposure. Phosphorylated Cx43 is represented by the upper P1 and P2 bands. The unphosphorylated form is represented by the lower band or P0. Figure 3-4 C). A statistically significant reduction in the Cx43 ratio was seen in the 5% ESC group as compared to the control group. There were similar statistically significant reductions in phosphorylated Cx43 with ESCs CM.

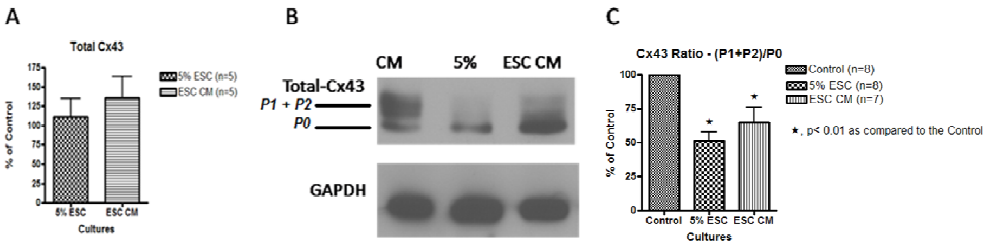

Figure 3-4- Cx43 expression. (A) Total Cx43 expression as a percentage of the control. (B) Western Blot for Cx43 expression. There was an increase in un-phosphorylated Cx43 in treatments as compared to the control. (C) The ratio of phosphorylation (P1+P2)/un-phosphorylated (P0) Cx43 of 5% ESC and ESC CM as compared to the controls (*, $p < .05$).

3.3 Cardiac Na^+ Channels are Downregulated in Treatment Conditions as Compared to Control

Cardiac Na^+ channel expression was significantly decreased upon exposure to ESCs or CM (Figure 3-5 A). Na^+ channel expression was 64.9 ± 6.0% in the ESC group of the control (n=8) and 73.8 ± 13.8 % of the control (n=7) in the ESC CM group (Figure 3-5 B). Na^+ channel expression was significantly reduced in both 5% ESC and ESC CM cultures.

Downregulation of the SCN5a gene transcription was seen in both the 5% ESC and ESC CM groups, using real time PCR (Figure 3-6). The SCN5a mRNA abundance in the 5% ESC group was 19.8 ± 10.0% (n=3) of the control and 48.8 ± 10.3% (n=3) of the control in the ESC CM group.

A concomitant downregulation of Na^+ current was observed. (Figure 3-7A). There was a statistically significant decrease in the peak Na^+ current from 47.7 ± 2.3 pA/pF (n=10) in the controls to 29.4± 1.7 pA/pF (n=10) in the ESC CM. Peak conductance was reduced by 38% (Figure 3-7 B). There were no statistically significant changes in steady state activation or inactivation, however. Therefore, exposure of cardiomyocytes to ESCs resulted in reductions in Na^+ channel mRNA, protein, and current.

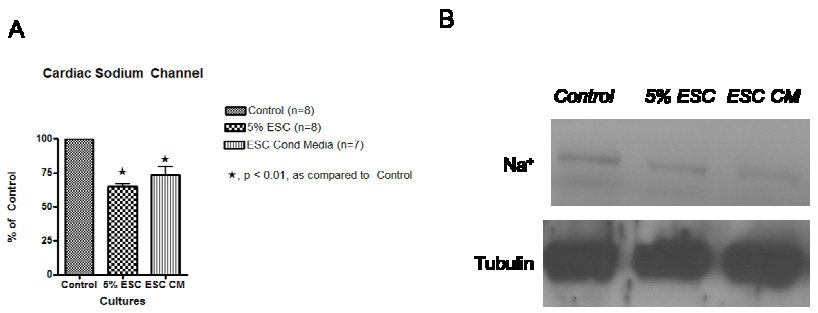

Figure 3-5 - Cardiac Na^+ channel. (A) Reduction in Na^+ channels in treatments as compared to Control (*, $p<0.01$) (B) Western Blot showing the decrease in Na^+ channel expression in both 5% ESC and ESC CM conditions .

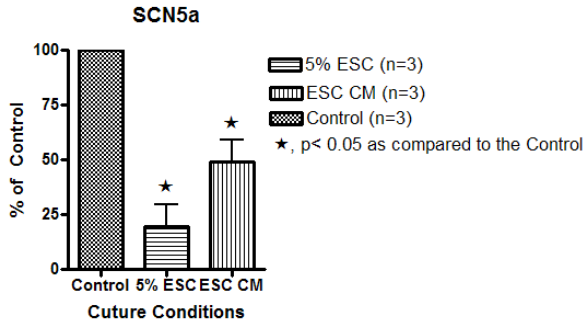

Figure 3-6 – RT-PCR of SCN5A. Reduction in Na+ channels in treatments as compared to Control (*, $p<0.05$)

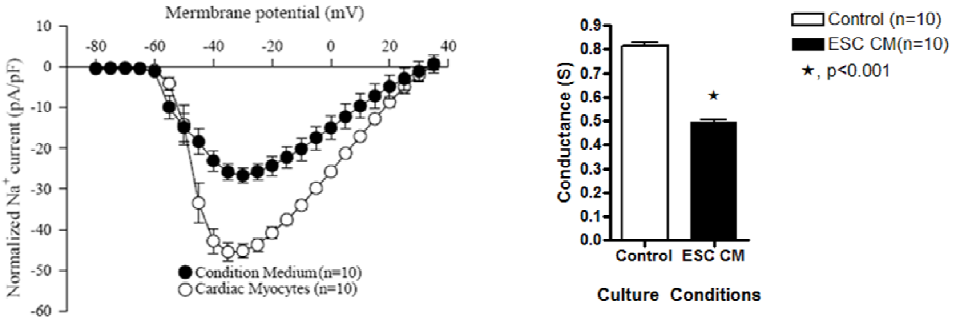

Figure 3-7 – Na^+ channel IV Curve (A) Average current voltage relationships for Na^+ current for the control and ESC CM groups. (B) Peak whole-cell Na^+ conductance

3.4 Modeling Suggests that the Molecular Changes Observed are Sufficient to Explain the CV Decrease

The LR model of the mammalian ventricular action potential was used to investigate the effects of molecular changes in connexins and Na^+ channels on CV. A 2D model consisting of individual cells connected through resistive gap junctions was implemented. A representative sequence of depolarization (red) and repolarization (blue) depicting intermediate membrane potentials of the myocytes using the simulation is illustrated in Figure 3-8. APs were initiated by an external stimulus applied to the middle of the 2D grid. Since uniform coupling was assumed, APs propagated uniformly in all directions (Figure 3-8).

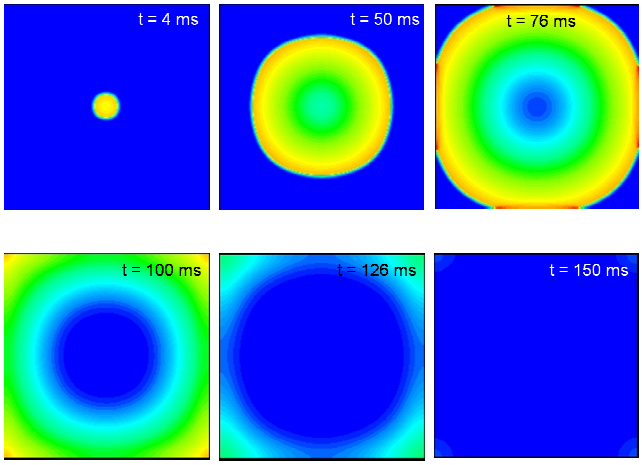

Figure 3-8 – Simulation of spontaneous depolarization of myocytes. There is uniform propagation in all directions.

The effect of changes in connexin and Na^+ channel expression seen in experiment were simulated by reducing g_j and G_{Na} by the fixed percentages shown in Figure 3-9. The model predicted a 51% and a 41% decrease in CV for the 5% ESC and the ESC CM groups,

respectively MEA data showed a 67% and a 70% decrease in CV for the 5% ESC and the ESC CM groups respectively, and similar changes were observed in the model. ISI and APD were not studied using the model. Figure 3-9 is a summary of the findings from the simulation; it suggests that the reductions in Na^+ current and connexin expression were sufficient to explain the majority of the reductions in CV in the treated cultures. In the model, the Cx43 reductions were based on the changes in phosphorylation patterns seen in the western blot analysis. It was assumed that P0 is a non functional form, while P1 and P2 are fully functional.

Result Type	Experimental			Simulation		
Group	**Control**	**5% ESC**	**ESC CM**	**I**	**II**	**III**
Changes	N/A	45% ↓ I_{Na} 49% ↓ Cx	27% ↓ I_{Na} 45% ↓ Cx	N/A	45% ↓ I_{Na} 49% ↓ Cx	27% ↓ I_{Na} 45% ↓ Cx
CV (cm/s)	14.23	4.56	4.18	15.5	7.53	9.14
% Change	0%	↓68%	↓70%	0%	↓51%	↓41%

Figure 3-9 – Comparison of the experimental CV and simulation CV. CV of the control in the model was taken as 15.5 cm/sec.

Change Condition	**I_{Na}**		**Connexin**	
% Change (Condition)	**25% ↓**	**50% ↓**	**25% ↓**	**50% ↓**
% Change Conduction Velocity	**11% (13.78)**	**26% (11.45)**	**16% (12.95)**	**37% (9.81)**

Figure 3-10 – Effects of Na^+ channel and connexin changes on CV. Based on simulations, connexin changes have a larger effect on CV than Na^+ channel.

The simulations were conducted by incorporating the observed changes in connexin and Na^+ channel expression in the model without including ESC cells. Therefore, additional simulations were conducted in which the role of incorporating cells with "ESC like" properties randomly in the 2D grid mimicking in vitro conditions. In order to achieve this, a random number generator algorithm was used to identify cells within the 2D grid that were subsequently converted to "ESC-like" cells. Since very little information is available regarding the electrophysiological properties of ESCs, they were simulated by clamping the membrane potential of the randomly identified cells to -35 mV.

The consequences of converting a fixed percentage of cells to ESC-like cells were also tested. A representative sequence of events occurring after conversion of 0.25 % of all the cells (100 of 40,000) to ESC cells is shown in Figure 3-9. Increasing the number of ESC-like cells (to 200) decreased the probability of a successful AP propagation following an external stimulus. This was mainly due to interactions between the depolarized ESC-like cells and the normal (repolarized) surrounding tissue, which made the tissue refractory due to inactivation of Na^+ channels.

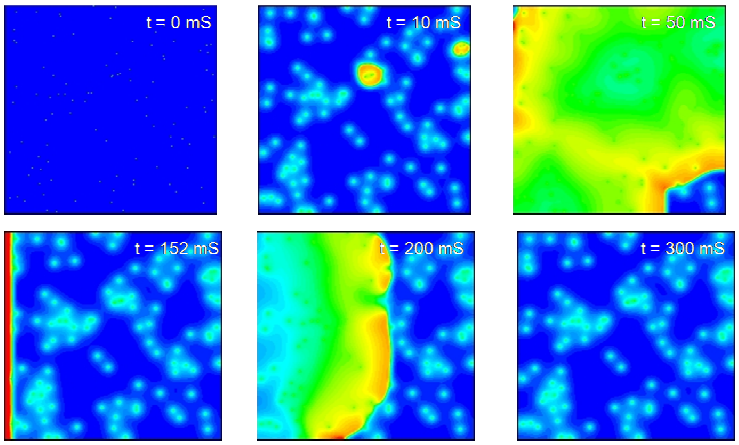

Figure 3-11 – Simulation to investigate role of ESCs on the spontaneous depolarization of myocytes. Each point corresponds to one cell with red being depolarized (>10 mV) and blue representing the resting potential (<-84mV). The top 3 panels show spontaneous depolarization. The bottom three panels show an AP initiated at the left column (after the initial spontaneous depolarization) propagating relatively uniformly from left to right. 0.25% of the cells were converted to ESC by clamping the membrane potential to -35 mV.

CHAPTER 4

DISCUSSION

4.1 Review of Findings and Implications

We have shown that ESCs have an effect on cardiomyocyte electrophysiological behavior. In our experiments, cardiomyocytes cultured with ESCs showed longer FPD (Figure 3-1), increased ISI (Figure 3-2), and a reduced CV (Figure 3-3). These changes correlated with reductions in phosphorylated Cx43 and Na^+ channel expression. The finding that ESC CM had the same effect on electrophysiological properties as ESC co-cultures implies that the results seen are because of paracrine factors and not physical contact between ESCs and cardiomyocytes.[60]

To better understand what was causing the changes, we examined the molecular determinants of CV further. There are two major determinants of CV, Cx43, which is responsible for intercellular communication, and Na^+ channels, which generate current for conduction. Protein analysis revealed a decrease in phosphorylated Cx43 (Figure 3-4 C) and cardiac Na^+ channel expression (Figure 3-5 A). RT-PCR showed a corresponding reduction in SCN5A mRNA abundance in ESC CM treated cells but no changes in Cx43 mRNA, suggesting that paracrine factors caused transcriptional changes in Na^+ channel expression and post-transcriptional changes in Cx43 phosphorylation. Assuming that dephosphorylated Cx43 did not participate in functionally active connexons, the simulations studies suggested that these changes were sufficient to explain the majority of the conduction slowing observed.

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Deleted: The fact that these results were observed in both co-culture and CM experiments suggests that secreted factors were responsible for the decrease in phosphophrylated Cx43 and fewer Na^+ channels that led to reduced gap junction communication. Further investigation was conducted to understand if these results were due to transcriptional or translational changes.

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Deleted: Decreases in Na^+ channel expression in ESC CM treated cells led to a significantly lower Na^+ current as compared to untreated cells. Based on simulation studies

Deleted: the decreases in both Cx43 phosphorylation and Na^+ current were the major factors that caused the drastic reduction of CV seen in co-culture and CM experiments.

4.2 Cardiomyocyte Cx43 Phosphorylation is Altered by ESC Paracrine Factors

Cx43 is a phosphoprotein, and changes in connexin phosphorylation have an affect on channel properties.[61] Phosphorylation of Cx43 appears to play a key role in channel assembly and the determination of channel conductance properties. Under normal conditions, almost all of Cx43 is found in the phosphorylated form.[62] Cx43 dephoshorylation is caused by a response to chemical agents or pathophysiologic conditions like myocardial ischemia which promotes uncoupling. Dephosphorylation Cx43 has been shown to reduce gap junction communication [80] and subsequently CV reduction in myocytes. Since there was no change in total Cx43, changes in phosphorylation patterns may be responsible for the observed changes in myocyte behavior. In ischemic hearts, immunoblot analysis showed that the total amount of Cx43 remained constant but showed an increase in desphysophorylated Cx43.[63]

Cx43 phosphorylation is controlled by a balance of kinase and phopshatse activities. Cx43 is associated with phosphatses that can regulate activity. Nevertheless, the lack of effect that we observed with okadaic acid, a phosphatase inhibitor suggests that the lack of phosphorylation observed is likely secondary to reduced kinase activity. Among those kinase known to phosphorylate Cx43 are mitogen-activated protein kinase (MAPK) or protein kinase C (PKC) has been associated with decreased gap junction intercellular communication.[65] Studies have suggested that disruption in gap junction communications were due to activation of an phosphatidylinositol 3-kinase/Akt pathway.[66]

In addition, Doble et al.[67] demonstrated that fibroblast growth factor 2 (FGF-2) led to the activation of PKC, which brought about changes in the phosphorylation state of Cx43. They also showed that FGF-2 does not cause changes in Cx43 mRNA.[67] Even though, FGF-2 induced

Comment [SCD3]: What is the point of this sentence?

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Comment [SCD4]: If these phosphorylate but render the channels inactive, they do not fit with your data.

Comment [SCD5]: Increases or decreases? How does this fit with your data? What is the point of this paragraph. Are there other candidates for the paracrine factor?

increases in phosphorylated Cx43 on a Threonine residue, this study suggests that there might be other factors that do not change Cx43 mRNA but affect phosphorylation patterns.

4.3 ESC Conditioned Media Induces Transcriptional Changes in SCN5A

Based on the concordance of RT-PCR, Western blot, and current data, it appears that ES cell exposure results in transcriptional downregulation of the Na^+ channel in cardiomyocytes. The promoter region of the channel has been characterized. Based on agents known or thought to be secreted from stem cells, possible mediators include…

For example, in cultured rat cardiac myocytes, adding Na^+ channel blocker mexiletine increase Na^+ channel mRNA expression and lead to an increased number of Na^+ channels.[68] Conversely, the addition of Ca^{2+} ionophore A23187 caused increases in intracellular calcium, which in turn lead to a fivefold decrease in Na^+ channel expression, while the addition of the calcium chelator BAPTA-AM had opposite results.[69-71]

This effect is consistent with that of cytokines known to transcriptionally regulate expression of other ion channels proteins including calcium regulating proteins (SERCA2), calcium releasing channel (CRC) and L type Ca^{+2} channels through gene expression at the transcript level.[72,73]

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Deleted: is transcriptionally regulated whereas Cx43 changes seem to be post transcriptional. There is also evidence that Na^+ channel transcription can be dynamically regulated.[68]

Comment [SCD6]: References.

Deleted: In general, mutations in the coding region of an ion channel gene may alter the gating kinetics of the channel and affect its expression level and distribution in the plasma membrane. This could lead to a defect in trafficking along the secretory pathway leading to further degradation of plasma membrane-associated ion channel proteins.

Comment [SCD7]: How does this explain your data? You must always return to your data.

Deleted: The influence of $[Ca]_i$ on the stability of Na^+ channel mRNA is currently unknown but changes in cardiac Na^+ channel mRNA have been linked to concurrent changes in $[Ca]_i$ in pathological circumstances.[68]

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Comment [SCD8]: Or you could use this data to suggest mediators of the effect.

Comment [SCD9]: Are the effects of these cytokines in the range of what you observed? Are there binding elements in the promoter? Could calcineurin activation explain the Cx and Na^+ channel effects?

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Deleted: is possible that paracrine factors in the media have elevated concentrations of cytokines leading to the following chain of affects: changes in $[Ca]_i$, reduced Cx43 mRNA expression, fewer Na^+ channels, and reduced I_{Na} results in CV reduction.

4.4 Co-Culture of Other Cell Types with Cardiomyocytes Has Similar Paracrine Effects

Others have seen effects of cells on native CMs. LaFromboise et al.[74] demonstrated that co-culture of fibroblasts with cardiomyocytes resulted in changes in myocyte structure and functional characteristics. Hypertrophy, intracellular expression of vimentin, and reduction of chronotropic contractile activity were induced. Research shows that hypertrophy leads to AP prolongation and increased I_{Ca}.[75] Myocytes cultured with CM from feeder-cells have also been shown to exhibit hypertrophy through the activation of different protein kinase pathways.[76] In initial studies (data not shown), we saw similar effects in fibroblast-CM, implying that the effect of ESCs may not be unique. Researchers also saw increases in the levels of vascular endothelial growth factor (VEGF), growth-regulated α-protein (GRO/KC), monocyte chemoattractant protein-1 (MCP-1), leptin, macrophage inflammatory protein-1 α (MIP-1 α), IL-6, IL-10, IL-12p70, and IL-17, and tumor necrosis factor (TNF)- α in their fibroblast conditioned media.[74] If our cells have a similar secretory profile, then there are several possible candidates to mediate the paracrine effects. For example, VEGF has been shown to cause Cx43 remodeling, which leads to disruptions in gap junction intracellular communication (GJIC). Studies have also shown that TNF- α causes downregulation of Cx43.[77] The effects of the other factors on the electrophysiological properties of cardiomyocytes still need to be further investigated.

Using optical mapping, Chang et al.[78] demonstrated that CV was reduced in co-cultures of MSCs and cardiomyocytes as compared to controls. The effects were dose dependent. Reentrant arrhythmias were induced in 86% of co-cultures containing 10% and 20% but not in controls or co-cultures containing only 1% MSCs.[78] We also observed no significant electrophysiological changes in co-culture studies with 1% ESCs as compared to controls, suggesting similarities between our studies and implying the effect on conduction velocity may

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Comment [SCD10]: What does this mean?

Comment [SCD11]: Were your cells the same or different. If different, why? Are the Cx and Na^+ channel changes consistent with hypertrophy?

Comment [SCD12]: If you bring this up, you will need data on whether yours are hypertrophied. How does this relate to your data?

Comment [SCD13]: Why are we not showing it?

Comment [SCD14]: What about the Abraham data?

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Deleted: addition, IM experiments were conducted with media that was kept in an incubator overnight to control for culture conditions as the possible cause of reduced myocyte function. These experiments revealed no significant deviation from the control studies suggesting that the effect is due to a secreted factor rather than incubation conditions.¶

Comment [SCD15]: This is good, but in the wrong place. Here you are talking about how your cell effects are the same or different from what others have seen.

not be unique to one type of stem cell.. The MSC studies also demonstrated that co-cultures exhibiting decreased CV are predisposed to reentrant arrhythmias. Their study suggested that reduced CV was due coupling of cardiomyocytes with unexcitable MSCs.[78] This is different from our study in the flowing ways as our ESC CM experiments suggested that without unexcitable ESCs in our culture, there were similar reductions in CV. MSCs are known to secrete growth factors and cytokines including macrophage, granulocyte, and granulocyte-macrophage colony stimulating factors, stem cell factor 1, leukemia inhibitory factor, stromal cell–derived factor-1, Flt-3 ligand, and IL-1, -6, -7, -8, -11, -14, and -15.[79] The authors speculate that the proarrhythmic risk of MSCs were due to these secreted factors.[78] Since ESCs are also unexcitable and our experiments suggest that paracrine factors are responsible for the altered myocyte behavior, it is possible that ESCs secrete similar growth factors that cause proarrhythmic behavior.

Comment [SCD16]: Did you show this data?

Comment [SCD17]: What other similarities or differences were observed? Was the mechanism the same? Did Cxs and Na^+ channels change in their studies? Do we know?

Comment [SCD18]: Are either of these effects going to be clinically significant, since clinical trials show no increased arrhythmias with MSCs? Is this because of lack of durability or because the changes do not matter?

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Deleted: results of reduced CV in both MSC and ESC co-cultures suggest that the effect of ESCs is not unique and that multiple cell types have a capability to alter cardiomyocyte behavior

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Comment [SCD19]: Reference? Or is this a continuation of the thought above?

Deleted: Since our experiments showed that exposure of cardiomyocytes to ESCs led to a decrease in CV, arrhythmias could also result from the introduction of ESCs to cardiomyocytes.

Comment [SCD20]: This needs to be grouped with the discussion on which factors could cause the effect. Then, you need to explore if any of these are known to cause changes similar to what you saw.

Comment [SCD21]: Why? How is that similar or different to what we saw?

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Comment [SCD22]: Ok, buit you are back to factors. You might want to discuss what paracrine factors may be doing this. What intracellular mediators are activated by these factors? Are any of these known to cause ion channel changes similar to what you saw?

4.5 Relevance of LR Model for APs

Modeling suggested that the observed Na^+ channel and Cx43 changes were sufficient to explain the reduced CV. Based on the simulation, it appears that both Cx43 and Na^+ channel are equally responsible for the reduced CV (Figure 3-10).

A possible limitation of the AP model used was that it did not take into account the presence of the stem cells. Nevertheless, the statistically indistinguishable effect of CM and the similar findings with Na^+ channels and CXs suggests that the cells that introduction of a small number of inexcitable cells is not likely to change the model conclusions dramatically. In addition, due to modeling constraints, the model uses only about 400 cells whereas our cultures have over 2 million cells. Since uniform conduction is presumed, it is possible to assume that the simulation is a small subset of the whole culture. Slight discrepancies between the experimental result and the model may have resulted because the model is based on a adult porcine channel parameters, while our cardiomyocytes are from neonatal rats.

Also, certain simplifying assumptions were made to make the modeling more tractable. Connexins were modeled as simple resistors and changes in Cx expression was modeled by changing resistance (Rg). The Na^+ channel changes were modeled by reducing the maximal conductance of the Na^+ current (G_{Na}) by fixed percentages. Finally, the data and modeling do not speak to the origin of the changes in ISI or FP duration.

Deleted: With the reductions in CV seen in our cultures, it was important to determine if the results we saw were based on the changes in Cx43 and Na^+ channel alone or if there were other factors responsible for causing such a reduction

Deleted: We used an established model for APs to further investigate the effects of the reduced expression of both connexins and cardiac Na^+ channels on CV. The LR Model was chosen because it correctly simulates the interaction of excitation and repolarization,[56] which is important because reentrant arrhythmias have this type of interaction between the head and tail of the reentrant AP.[80] The results of the simulation suggest that changes in Cx43 and Na^+ channel drastically alter the myocyte behavior.

Deleted: The simulation and experimental results are not equivalent but they are comparable. While there may be some other factors that affect CV, this simulation helped identify the major factors.

Deleted: in this study is that uniform conduction throughout the culture is assumed, which may not always be the case. Also, the model has a single activation site, which is not always the case as some cultures have multiple activation sites. These limitations, however, minimally affect our results.

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Deleted: Also, in this study neonatal cells were used, which have higher beating rates than adult cells. These assumptions are reasonable, since the purpose of this study was to see if the results of our experiments were plausible and not to determine exact values.

Deleted: Even though the results obtained with the model are not exactly the same as seen in our MEA data, similar trends were observed. The limitations previously mentioned may partially explain the discrepancy. While Cx43 and Na^+ current are major determinants of CV, there are other factors that affect conduction, like calcium concentration, that have not been considered in the model.

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4.6 Limitations, Future Directions and Conclusions

This study showed that the introduction of ESCs leads to alterations in cardiomyocytes that could be arrhythmogenic, but did not demonstrate arrhythmia formation directly. The study did however show that addition of ESCs in vitro cause cardiomyocytes to display pro-arrhythmic phenotype with longer APDs and reduced CV. Future experiments using an in vivo model will be required to demonstrate arrhythmia development in response to ESC transplantation. Our data do not speak to the effect of ESC-derived CMs on native heart cells or of CMs on ESC differentiation. These needs to be further investigated. Also, these were relatively short term experiments. Since the goal of in vivo cell transplantation is to have long term engraftment, the long term effects on cardiomyocytes may differ. Moreover, our studies do not take into account the effects of altered immune response as part of cell replacement.

Results may differ by species. Our studies look at the effect of mouse ES cells on rat CMs. Future studies will need to look at a same species model.

Finally, although Cx43 is the most widely available gap junction protein found in ventricular cells, there are trace amounts of Cx40. Studies have shown increased amounts of Cx40 is found in failing ventricular heart tissue.[82] Increasing the ratio of Cx40 to Cx43 has been shown to reduce propagation velocity [83] and there is speculation that the Cx40 might have an effect on working ventricular myocyte junctions.[84] Future studies may need to look at the effect of Cx40 on propagation velocity.

In conclusion, ESCs hold promise as a cellular therapy option because of their potency and renewability. At the same time, ESCs demonstrate arrhythmic potential and before ESCs

Comment [SCD23]: You probably need a chapter in the discussion on the relationship of CV to arrhythmia to show that the magnitude of changes that you see is consistent with an increase in arrhythmic potential.

Comment [SCD24]: Why? What can you do to overcome in the future?

Deleted: The in vivo model is essential in understanding the full effect of ESCs on the heart. There might be in-vivo factors like temperature, other cell types, proximity, immune response that play a role that can not be considered in an in-vitro model. ¶

Deleted: The effect of ESC differentiation on cardiomyocytes has not yet been studied and

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Deleted: At the time the experiments were conducted, there was no readily available rat ESCs. Therefore, we used xenogenic mouse ESCs with the rat neonatal cardiomyocytes. Since there limitations in xenogenic cells like risk of disease and immune acceptance, future studies should focus on using an allogenic model. There are differences between animal and humans that need to be considered. For example, the human heart beats 10 times slower than that of mice and human ventricular ejection fraction is substantially lower.[81]

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can be used as a cell source, their interaction with the native myocardium should be further investigated.

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APPENDIX A

PROCEDURE OF NEONATAL CARDIOMYOCYTE ISOLATION

Day 1: Perform on ice.

1. The beating hearts from 1- to 2-day-old Sprague-Dawley rats were surgically removed under anesthesia, and immediately place the heart in the centrifuge tube, which contained 35 ml sterile calcium- and magnesium-free Hank's Balanced Salt Solution (pH7.4), to chill and rinse. Swirl the tube to rinse hearts, and then pour off most of liquid. Rinse the hearts with 10 ml of calcium- and magnesium-free Hank's Balances Salt Solution, pour off the liquid as before, and then transfer the hearts to the Petri dish. Remove the atrium and mince the tissue of ventricles with small scissors to less than 1 mm^3, pieces keeping tissue at 0 ºC.
2. Add calcium- and magnesium-free Hank's Balanced Salt Solution to Petri dish to a final volume of approximately 9 ml.
3. Transfer 1 ml of the contents of the trypsin into the Petri dish and mix completely by swirling. Final trypsin concentration is 50 µg/ml.
4. Place the lid on the Petri dish and immediately place in refrigerator overnight (16-20 hours) at 2-8 ºC.

Day 2

1. Remove Petri dish from refrigerator and bring to tissue culture hood on ice. Transfer tissue to 50 ml centrifuge tube on ice using wide-mouth pipette.
2. Transfer 2000 µg Trypsin Inhibitor in 1 ml calcium- and magnesium-free Hank's Balanced Salt Solution into tube and mix.
3. Oxygenate tissue for 1 min by passing oxygen over the surface of the liquid.
4. Warm tissue in water bath at 37 ºC for 30 min.
5. Slowly transfer 1500 Units of Collagenase in 5 ml Leibovitz L-15 Media into tube and mix. Cap tube tightly.
6. Place tube on slowly rotating instrument (Techne Hybridizer HB-1D) at 37 ºC and incubate for 45 min.
7. Remove tube from incubator and return to tissue culture hood. Triturate about 10 times to release cells with standard 10 ml plastic serologic pipette.

8. Rinse a Cell Strainer (BD Falcon, REF 352350) with 1 ml of the L-15 culture medium. Allow tissue residue to settle 4 min, then (with same pipette) filter the supernatant through the Cell Strainer into a fresh 50 ml centrifuge tube.
9. Add 5 ml additional L-15 culture medium to tissue residue, repeat trituration step. Allow tissue residue to settle as before, then filter cells through the same Cell Strainer. Rinse mesh gently with 2 ml L-15 culture medium, oxygenate cells 1 min, then allow filtered cells to remain undisturbed about 20 min at room temperature.
10. Swirl cells gently, and remain undisturbed 10 min at room temperature. Sediment cells at 1000rpm for 3 min.
11. Suspend final cell pellet in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Corp. Carlsbad, CA). Pipette gently to disperse. Count the cells using a hemacytometer, and estimate cell viability by using Trypan Blue exclusion procedures, then pre-plate cells in 150 mm tissue culture dish. Place dishes in a 37 ºC incubator for 90 min in order to reduce the contamination of cardiac fibroblast.
12. Take out the dishes from the incubator, transfer the unattached cells into a 50 ml centrifuge tube. Sediment cells at 1000rpm for 3 min, suspend cell pellet in DMEM with 10% FBS, count alive cell number.
13. Plate myocytes on the multi-electrode arrays (MEA) (pre-coated with 0.1 % Gelatin) at a density of 2×10^6, and on T-25 flasks.

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This is a simplified method of simulating changes in Cx and Na^+ channel expression. In the LR model, however, that is how Na^+ channel and Cx expression are modeled. These interactions, in vivo, are likely to be more complex than in our model. This simplification could explain the slight variation seen between the model and the experimental results.